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Keratin Classes: Molecular Markers for Different Types of Epithelial Differentiation

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Keratins are a group of water-insoluble proteins (molecular weight range 40–70 K) that form 10-nm tonofilaments in a wide variety of epithelial cells. The subunit composition of the keratin filaments varies with cell type, period of embryonic development, stage of histologic differentiation, cellular growth environment, and disease state. To better understand the functional significance of individual keratin species, we have generated three monoclonal antikeratin antibodies to different subsets of keratins and used these antibodies to localize specific keratins in normal human epidermis by a combination of immunohistochemical and biochemical techniques. The results indicate that the 50 K and 58 K keratins are present in all cell layers including the relatively undifferentiated basal layer, whereas the 56.5 K and 65–67 K keratins are associated only with the more differentiated cells above the basal layer. In a separate series of experiments, we used the monoclonal antibodies to survey the keratins expressed by various

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Abbreviations:
CB: Coomassie Blue
PAGE: polyacrylamide gel electrophoresis
PAP technique: peroxidase-antiperoxidase technique
SDS: sodium dodecyl sulfate

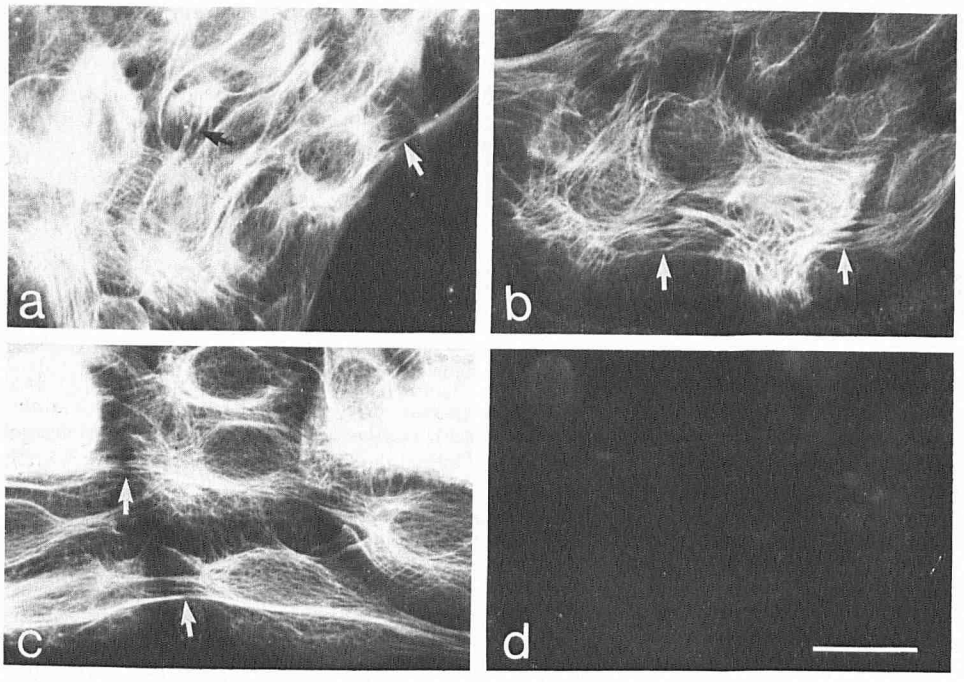

FIG. 1. Indirect immunofluorescence staining of cultured human epidermal keratinocytes with monoclonal anti-keratin antibodies. Tertiary cultures of human epidermal cells derived from newborn foreskin were grown on glass coverslips, fixed with cold methanol, and stained with mouse antibodies by indirect immunofluorescence: (*a*) AE1 antibody, (*b*) AE2 antibody, (*c*) AE3 antibody, and (*d*) P3 control. Arrows indicate cell-cell junctions presumably containing desmosomes. Bar = 25 μm. (Reproduced from the *Journal of Cell Biology* 95:580, 1982, by copyright permission of the Rockefeller University Press.)

nonepidermal epithelia. The data show that keratins can be divided into at least seven major classes according to their immunologic reactivity and size. Among the keratin classes, the 50 K and 58 K classes appear to be characteristic of all stratified squamous epithelia, whereas the 56.5 K and 65–67 K classes are unique to the keratinized epidermis. These findings suggest that specific keratin classes, as defined by monoclonal antibodies, may serve as useful markers for different types of epithelial differentiation (simple versus stratified, keratinized versus nonkeratinized).

Recent studies have demonstrated that keratin-type intermediate filaments (10 nm in diameter) are present in almost all epithelial cells, both in vivo and in culture, but not in any nonepithelial cell types, such as fibroblasts, muscle cells, astrocytes, or neurons [1–4]. Thus keratin represents an important marker for the unambiguous identification of cells with an epithelial origin.

Compared with other types of intermediate filament proteins (vimentin, desmin, neurofilament, and glial filament), keratin is very complicated in terms of subunit composition (for a recent review on intermediate filaments, see [5]). In vitro reconstitution experiments suggest that at least two keratin species are required for filament formation [6,7]. Biochemical analyses indicate that epithelial keratin composition varies depending on cell type [8–11], period of embryonic development [12–14], stage of histologic differentiation [15–20], cellular growth environment [8,9,21–26], and disease state [27–35]. It is therefore perhaps not surprising that numerous keratin species have been described in the literature. However, although it has been amply demonstrated that keratin expression can be influenced by multiple factors, the existing data do not reveal any precise rules concerning the expression of individual keratin proteins. The biological meaning of keratin heterogeneity thus remains largely obscure.

In this paper we review some of our results from several experiments designed to provide data that shed light on the possible functional significance of individual keratin species. Using monoclonal antibodies as a tool, we have localized specific keratins in human epidermal layers [18] and surveyed keratins expressed by various in vivo mammalian epithelia [36]. Our results indicate that keratins can be grouped, based on their immunoreactivity with monoclonal antibodies and their molecular weight, into a relatively small number of classes whose expression follows rules related to epithelial morphology (simple versus stratified, keratinized* versus nonkeratinized). Specific keratin classes may therefore be regarded as molecular markers for different types of epithelial differentiation.

MONOCLONAL ANTI-KERATIN ANTIBODIES

Using SDS-denatured human callus keratins as the immunogen, we prepared three mouse monoclonal anti-keratin antibodies (designated AE1, AE2, and AE3) by the hybridoma technique [18]. In cultured human epidermal cells, all three antibodies decorated a wavy network of cytoplasmic fibers associated with desmosomal cell-cell junctions (Fig. 1). In tissue sections, the three antibodies reacted specifically with the epidermis (see below) and other epithelial tissues, but not with any nonepithelial cell types. These results strongly suggest that the antibodies are specific for keratin-type intermediate filaments [18,36].

The reactivity of the antibodies to individual keratin polypeptides was determined by the immunoblot technique [18]. Keratins were isolated from the living layers of human abdominal epidermis by boiling the water-insoluble tissue residues with 1% sodium dodecyl sulfate (SDS) in the absence of a reducing agent [22]. Analysis of the extract by SDS polyacrylamide gel electrophoresis (PAGE) revealed four major polypeptides with apparent molecular weights of 50 K, 56.5 K,† 58 K, and 65–67 K [18] (Fig. 2, *lane CB*). For immunoblotting, these polypeptides were transferred electrophoretically from an unstained polyacrylamide gel to nitrocellulose paper, which was then stained with individual monoclonal antibodies by the peroxidase-antiperoxidase (PAP) technique. The results indicated that AE1 antibody specifically bound to the 50 K and 56.5 K keratins (Fig. 2, *lane 1*), AE2 bound to the 56.5 K and 65–67 K keratins (*lane 2*), and AE3 bound to the 58 K and 65–67 K keratins (*lane 3*). Control experiments showed that culture medium conditioned by P3 myeloma cells did not bind to any epidermal proteins. These results indicate that common antigenic determinants are present in various subsets of keratins

* As defined morphologically by the formation of membrane-coating granules, keratohyalin granules, and enucleated horny cells.

† Previously described as 56 K keratin [18,36].

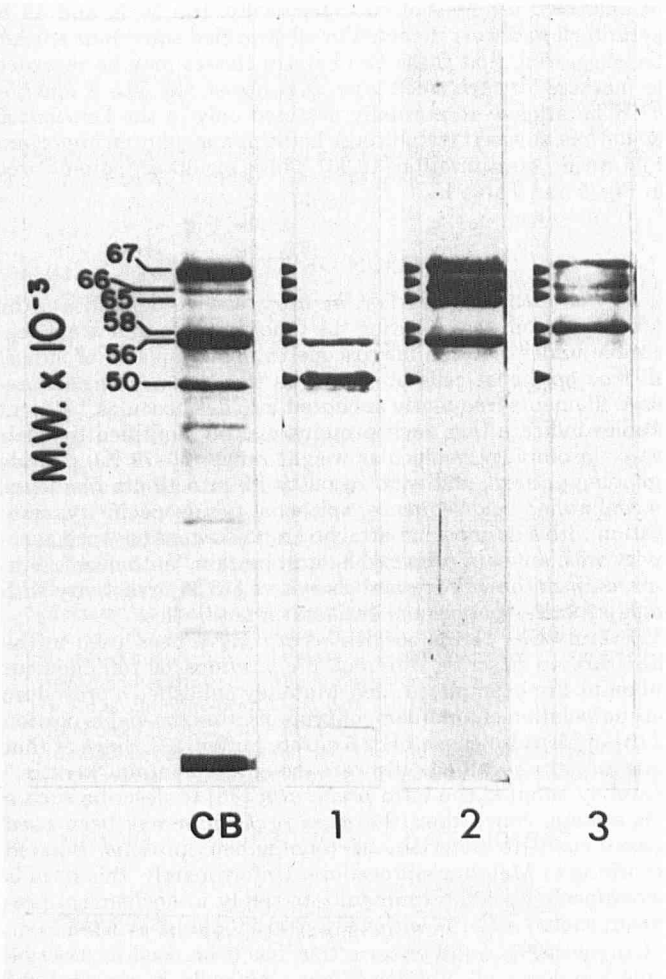

FIG 2. Binding of monoclonal antibodies to human epidermal keratins separated by SDS gel electrophoresis (immunoblot analysis). Water-insoluble cytoskeletal proteins extracted from the living layers of human abdominal epidermis were separated on SDS gels and either stained directly with Coomassie Blue (*CB*) or electrophoretically transferred onto nitrocellulose paper and then stained by the PAP technique with AE1 antibody (*lane 1*), AE2 antibody (*lane 2*), or AE3 antibody (*lane 3*). (Adapted from [18], with permission.)

and that the three antibodies, in combination, recognize all major epidermal keratins [18,36].

IMMUNOLOCALIZATION OF KERATINS IN HUMAN EPIDERMIS

Several types of experiments have been conducted to study the sequential expression of keratins during epidermal differentiation. By analyzing keratins extracted from horizontal sections of human skin by SDS PAGE, Fuchs and Green concluded that cells in the lower epidermal layers possess predominantly low-molecular-weight keratins, whereas cells in the upper layers expressed high-molecular-weight keratins in addition [9].

Taking an immunologic approach, Viac et al. [15] and Vidrich and Sun [16] have raised antisera against major human callus keratins partially purified by preparative SDS gel electrophoresis and used these antibodies to stain frozen skin sections by immunohistologic techniques. It was demonstrated that antisera prepared against the high-molecular-weight (65–67 K) keratins preferentially stain cells above the basal layer [15,16]. Since at least one of these antisera was shown to be specific for the 65–67 K keratins by the immunoblot technique [19], such a result, in conjunction with the horizontal-sectioning [9] and cell-fractionation data [20], strongly suggests that the high-molecular-weight keratins are associated with cells above the basal layer.

Antisera prepared against some other callus keratins also exhibited selective staining of epidermal layers. For example, antiserum to a "55 K" callus keratin was found to stain cells above the basal layer, leading to the suggestion that such a keratin occurs chiefly in the suprabasal, differentiated cell compartment [17]. In addition, antisera prepared against some other low-molecular-weight callus keratins stained either the entire epidermis [16] or preferentially the basal layer [17]. These data should be interpreted with caution, however, because of the potential problem of antigen "masking" (see below; [18]) and because immunoblotting data showed that some of these antisera crossreact with multiple keratin species [19].

Monoclonal anti-keratin antibodies provided additional data concerning the localization of individual keratins in human epidermis. The three antibodies (AE1, AE2, and AE3) produced strikingly different immunofluorescent staining patterns when applied to frozen sections of normal human epidermis. AE1 selectively stained the basal layer (Fig. 3*a*), AE2 stained only the suprabasal layers (Fig. 3*b*), and AE3 stained the entire epidermis (Fig. 3*c*). These results indicate that at least one of the keratin antigens recognized by AE1 antibody (50 K and 56.5 K) must be present in the basal layer. In addition, the observation that AE3 antibody—which recognizes both the 58 K keratin and the suprabasally located 65–67 K keratin—stained the entire epidermis suggests that the 58 K keratin is present in basal cells.

Although immunohistochemical staining provides useful information, it should be kept in mind that while positive staining establishes the presence of an antigen, the lack of staining does not prove its absence. That some keratin antigens might actually be masked and thus undetected *in situ* was suggested by the observation that AE1 and AE2 antibodies, both of which recognized the same 56.5 K keratin (Fig. 2), stained mutually exclusive parts of the epidermis (basal versus suprabasal; Fig. 3*a*, *b*). To investigate this possibility, we prepared serial horizontal sections of human heel epidermis [9] and analyzed their keratins by the immunoblot technique. The resolving power of monoclonal antibodies allowed us to follow detailed changes in specific keratin antigens during epidermal differentiation. Immunoblotting analysis using AE1 antibody clearly showed that the 50 K keratin was present in all epidermal living layers, including the innermost region; in contrast, the 56.5 K keratin was detected by AE1 (and AE2) only in sections containing upper epidermis [18]. AE3 staining data further showed that the 58 K keratin, like the 50 K keratin, was readily detected in all epidermal sections, including those enriched with basal cells [18].

Results from these immunohistochemical and biochemical studies suggest that the 50 K and 58 K keratins are present in all living layers, including the basal layer, whereas the 56.5 K and 65–67 K keratins are characteristic of suprabasally located, terminally differentiated cell layers. These results are schematically summarized in Fig. 4 [18–20].

TISSUE-DISTRIBUTION OF KERATINS

In order to gain information concerning the tissue-distribution of keratins, we prepared water-insoluble cytoskeletal proteins from a variety of mammalian epithelia and analyzed them by the immunoblot technique. Epithelia from monkey, human, and rabbit were used in these experiments, with similar results. The three monoclonal antibodies recognized in various epithelia a large number of keratin-related polypeptides, which can be divided into at least seven major classes according to their immunologic reactivity and molecular weight (40 K, 46 K, 50 K, 52 K, 56.5 K, 58 K, and 65–67 K; Table I). AE1 antibody reacted with the 40 K, 50 K, and 56.5 K keratin classes (AE1 family), whereas AE3 reacted with the 46 K, 52 K, 58 K, and 65–67 K keratin classes (AE3 family). Interestingly, AE2 re-

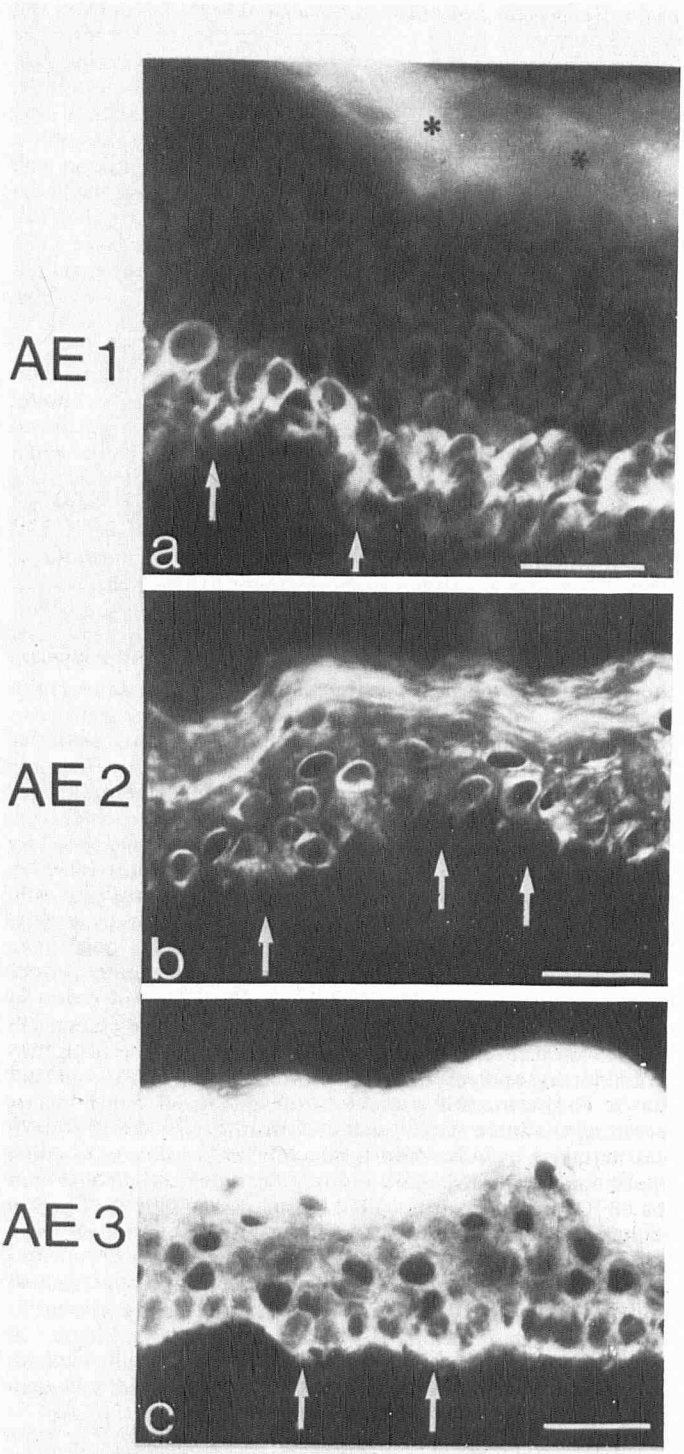

FIG 3. Immunofluorescence staining of frozen sections of normal human abdominal skin with monoclonal anti-keratin antibodies. *a*, AE1 antibody. Note the selective staining of basal cells. Arrows indicate the epidermal-dermal junction; asterisks denote the nonspecific staining of stratum corneum. *b*, AE2 antibody. Note the selective staining of suprabasal cell layers. *c*, AE3 antibody. Note the staining of the entire epidermis. Bar = 25 μm. (Adapted from [18], with permission.)

acted with the largest member of each family, i.e., the 56.5 K and 65–67 K keratin classes (Table I).

A comparison of the keratins expressed in various mammalian epithelia revealed a strong correlation between the expression of specific keratin classes and certain morphologic features of epithelial differentiation. Specifically, the 50 K and 58 K keratin classes were detected in all stratified squamous epithelia, suggesting that these two keratin classes may be regarded as markers for such a cell type. In contrast, the 56.5 K and 65–67 K keratins were normally detected only in the keratinized epidermis, suggesting that these keratins may play an important role during keratinization [18,36]. These results are summarized in Fig. 5 and Table I.

DEFINITION OF KERATINS

As was mentioned earlier, *keratins* can be defined as "the subunit proteins constituting the tonofilaments that are recognizable under electron microscope in the cytoplasm of almost all true epithelial cells or tissues as the 10-nm intermediate-sized filaments frequently anchored into desmosomes." Recent studies indicate that such proteins can be identified by their water-insolubility, molecular weight range (40–70 K), peptide mapping pattern, ability to reconstitute into 10-nm filaments, partial amino acid sequence, epithelial tissue-specificity, association with a desmosome-attached tonofilament network, reactivity with antisera prepared against certain "authentic" keratins, and, as shown here and elsewhere [18,36], reactivity with highly specific monoclonal antikeratin antibodies.

Several other terms besides *keratin* have been used in the literature to describe different preparations of tonofilament subunits. For example, in 1965, Matoltsy published a procedure for the isolation of tonofilament proteins from the viable portion of the epidermis using a pH 2.6 citrate buffer [38]. Since at that time only the cornified cells were thought to contain "keratin," Matoltsy adopted the term *prekeratin* [38] to describe such a preparation. Since then the term *prekeratin* has been used operationally to describe the tonofilament proteins isolated according to Matoltsy's procedure. Unfortunately, this term is sometimes used indiscriminately to imply a biochemical precursor; such a usage is misleading and should be avoided.

Cytokeratin is another term that has been used to describe some tonofilament proteins. Since classically it was thought that keratin-type proteins were unique to the epidermis and its appendages, Franke et al. coined the word *cytokeratin* to describe the keratin-like proteins of various nonepidermal epithelia [40]. However, recent results from both our group [36]

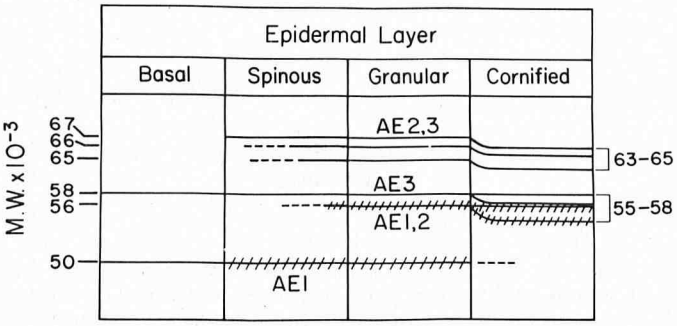

FIG 4. A schematic summary of changes in keratin expression during normal epidermal differentiation. A solid line denotes the presence of the keratin and a dotted line represents the possible presence of the keratin in the cell layer. A hatched line indicates that the antigen is present but undetectable by AE1 antibody in frozen sections. Breaks in lines between granular and cornified layers indicate partial degradation of keratins during stratum corneum formation [9,18]. As shown earlier, keratins of the cornified layer are cross-linked by intermolecular disulfide bonds and therefore can only be extracted after reduction [22,45]; however, keratins of the living layers (basal, spinous, and granular) are not cross-linked and are thus extractable with SDS alone [18] or with a pH 2.6 citrate buffer ("prekeratin" of Matoltsy [38, 47]). (Reproduced from the *Journal of Cell Biology* 95:580, 1982, by copyright permission of The Rockefeller University Press.)

TABLE I. *Keratin families and classes as defined by monoclonal anti-keratin antibodies*

Keratin family[a]	Keratin class (M_r)	Individual polypeptides	Charge[b]	cDNA[c]	Epithelial-cell type[d]			Expression in epidermis[h]	Marker for
					Simple[e]	Stratified			
						Nonkeratinized[f]	Keratinized[g]		
AE1	56.5 K[i]	56.5 (10 + 11)[j]	Acidic		−	−	+	Suprabasal	Keratinization[k]
	50 K	50 (14 + 15)	Acidic	pKB-2	−	+	+	Basal and up	Stratified epithelia
		48 (16)							
		46a (17)		pKB-2					
	40 K	40 (19)			+	+	−		
AE3	65–67 K[i]	67 (1)	Basic		−	−	+	Suprabasal	Keratinization
		66 (2)							
		65							
	58 K	58 (5)	Basic	pKA-1	−	+	+	Basal and up	Stratified epithelia
		57							
		56 (6)		pKA-1					
		55							
	52 K	52 (8)	Basic to neutral		+	+	−		
	46 K	46 (18)	Basic to neutral		+	+	−		

[a] Monoclonal antibodies [18,36].
[b] Determined by isoelectric focusing [18,41].
[c] The messenger RNAs for these keratins hybridize with cDNA clones pKB-2 and pKA-1 according to positive hybrid selection [43].
[d] Classified according to the normal histology of the major epithelial cell types.
[e] Simple epithelia: gall bladder, stomach, small intestine, liver, kidney, pancreas, and diaphragm.
[f] Stratified, nonkeratinized epithelia: esophagus, cornea, conjunctiva, thymus, bladder (transitional), and trachea (pseudostratified columnar).
[g] Stratified keratinized epithelium: epidermis.
[h] Based on immunolocalization data [18,19].
[i] Recognized by AE2 antibody [18,36].
[j] Numbers in parentheses indicate the tentative correlation of our nomenclature [18,36] with that used by Moll et al. [41].
[k] As defined morphologically by the formation of membrane-coating granules, keratohyalin granules, and completely enucleated horny cells.

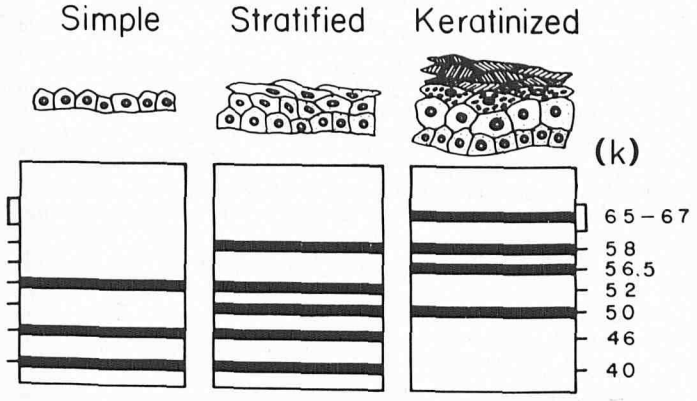

FIG 5. A schematic summary of the typical tissue-distribution of keratin classes, as detected by monoclonal antibodies using the immunoblot technique, in simple, stratified (nonkeratinized), and keratinized epithelia [36]. The 40 K, 50 K, and 56 K keratin classes are defined by AE1 antibody, while the 46 K, 52 K, 58 K, and 65–67 K keratin classes are defined by AE3 antibody (see Table I).

(Table 1) and Franke's group [41] have shown that epidermis and other epithelia share many common tonofilament subunits. For example, the 50 K and 58 K epidermal keratins are present in various quantities in all stratified epithelia [36,41]. Even the 56.5 K and 65–67 K keratins, which are normally found only in the epidermis, are not truly epidermis-specific, since they become readily detectable in corneal, conjunctival, and several other epithelia when the tissues become keratinized during vitamin A deficiency [42]. Conversely, certain tonofilament components normally found in almost all epithelia except the epidermis (e.g., the 40 K component [36,37]) are expressed by epidermal cells both in embryo [14; S.C.G. Tseng, unpublished] and in culture [R. Eichner, unpublished]. These data clearly indicate that the classification of tonofilament proteins into the two categories of (epidermis-specific) "keratins" versus (non-epidermal) "cytokeratins" can be problematic and impractical.

KERATIN FAMILIES

The three monoclonal antibodies (AE1, AE2, and AE3), in combination, recognize almost all known keratin species. These keratins can be divided into two mutually exclusive *families*‡ according to their reactivity with AE1 and AE3 antibodies (Table I). Keratins of the AE1 family are, in general, more acidic (isoelectric point less than 5.5) than those of the AE3 family (pI 5.5–8) [18,41]. In addition, the AE1 and AE3 families appear to correspond to the *pKB-2* and *PKA-1* keratin gene families, respectively, which have been identified in cultured human epidermal cells by DNA cloning [43]. Interestingly, at least one keratin from each family is expressed in all the in vivo epithelial tissues that we have examined so far. Our results thus strongly suggest that the concept of two keratin families applies not only to in vitro cultured cells [43], but also to in vivo tissues. Moreover, our data demonstrate that keratins of these two families are immunologically and biochemically distinct and can be conveniently defined by the use of monoclonal antibodies.

KERATIN CLASSES

Members of each keratin *family*, as defined by monoclonal antibodies, share at least one antigenic determinant and therefore must be structurally related. These related molecules can be further divided, according to their size, into keratin *classes* (Table I). At least two of the keratin classes contain multiple components whose expression varies depending on cell type, cellular growth, and morphologic differentiation. The 50 K keratin class of the AE1 family, for example, contains a 50 K component present in various quantities in all stratified epithelia, plus a 48 K and a 46 K keratin in hyperproliferative epidermal diseases (R.A. Weiss, unpublished observation), pilosebaceous tract [35], and cultured keratinocytes [21,22; R Eichner, unpublished observation]. Similarly, the 58 K keratin class of the AE3 family contains a 58 K component present in all stratified squamous epithelia, a 55 K keratin that appears to

‡ Or "subfamilies" if all keratins are collectively defined as a family.

be unique to the cornea, and a 56 K keratin expressed in hyperproliferative epidermal diseases as well as cultured keratinocytes [36,41].

The tissue-distribution data reveal that among keratin classes of each family, the smaller ones are widely distributed, whereas the larger ones are limited to more complex epithelia (Table I). Thus, within the AE1 family, the 40 K keratin is present in all epithelia except epidermis; the 50 K keratin class is present only in the more complex stratified squamous epithelia, but not in any simple epithelia; and the 56.5 K keratin is unique to the most complex keratinized epidermis [36]. A parallel situation exists in the AE3 family. The smaller 46 K and 52 K keratin classes are present in all simple and nonkeratinized stratified epithelia; the 58 K keratin class is present only in stratified squamous epithelia, but not in simple epithelial; and the high-molecular-weight (65–67 K) keratins are unique to keratinized epidermis [36]. Such a requirement for larger keratins by the more complex epithelia (Fig. 5) as well as the sequential expression of keratins during epidermal differentiation (Fig. 4) strongly suggest that different keratin classes may play different structural and/or functional roles during epithelial differentiation.

Moll, Franke, and coworkers have recently conducted a series of experiments in which they analyzed keratins of various human epithelial tissue by two-dimensional PAGE. Using such an approach, they have identified and characterized 19 keratin species with respect to their size, isoelectric point, peptide mapping pattern, and tissue specificity [41]. So far, it appears that their data and ours are in excellent agreement (Table I).

CONCLUSIONS

Using monoclonal antibodies, we have been able to define at least seven major keratin classes that belong to two families. Tissue-distribution studies revealed a strong correlation between the expression of specific keratin classes and some well-defined morphologic features of epithelial differentiation (simple versus stratified, keratinized versus nonkeratinized). These results emphasize the importance of epithelial morphology or structure as an underlying factor in keratin expression. Thus the 50 K and 58 K keratin classes may be regarded as molecular markers for all stratified squamous epithelia, whereas the 56.5 K and 65–67 K keratins may represent markers for keratinized epithelia. Such a concept provides a simple explanation for the changes in keratin expression observed under a wide variety of conditions that are accompanied by changes in epithelial morphology. These conditions or processes include embryonic development [12–14], variation in anatomic sites [9,44,45], disease state [27–35], and in vitro growth environment [8,9,21–26]. This concept may also have important implications for future studies of epithelial differentiation, for differential diagnosis of different types of carcinomas, and for determining the in vivo origin of cultured epithelial-cell lines.

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Specificity and Inhibition of the Epidermal Cell Detachment Induced by Pemphigus IgG in Vitro

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IgG isolated from sera of patients with pemphigus vulgaris (PV) has been shown to induce cell detachment when added to primary epidermal cell cultures (PECC). We studied the specificity of this phenomenon. IgG fractions were purified from the sera of five patients with PV and control IgG fractions from the sera of normal donors and patients with bullous pemphigoid (BP), systemic lupus erythematosus (SLE), and anti-AB blood group sera (anti-AB). IgG fractions were added to PECC either at initial plating (0 hours), at media change (48 hours), or sequentially at both times, and cell detachment was quantitated at 72 and 96 hours. Significant cell detachment occurred only when PV IgG was added to the growth media sequentially at 0 and 48 hours ($p = 0.001$), and this effect was dose-dependent for either dose. Substitution of an unrelated IgG (BP, SLE, or anti-AB) at either time points reduced cell detachment to near control values. Furthermore, cell detachment was inhibited by the addition of the proteinase inhibitors α_2 macroglobulin (70% inhibition of detachment), aprotinin (63% inhibition), soybean and lima bean trypsin inhibitor (62 and 64%, respectively), and pepstatin (49%), but not by the inhibitors chymostatin, leupeptin, or antipain. These data confirm that PV IgG induces increased cell detachment in PECC and shows that this effect is specific for PV IgG, is dose-dependent, and may be inhibited by certain proteinase inhibitors.

Pemphigus vulgaris (PV) is a severe and often fatal blistering disease affecting skin and mucous membranes in which intraepithelial vesicles form as a result of a distinctive process of cell detachment termed *acantholysis* [1]. The majority of patients with PV have IgG class autoantibodies against squamous epithelial cell surfaces [2]. These autoantibodies are detected both bound to the diseased epithelium and circulating in the serum,

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Abbreviations:
Monolayer: epidermal cell culture
PV: pemphigus vulgaris
BP: bullous pemphigoid
SLE: systemic lupus erythematosus
CF-II: Cohn Fraction II
ANA: anti-nuclear antibody
nDNA: native deoxyribonucleic acid
Ro: Ro or Sjogren syndrome-associated antigen
RNP: ribonuclear protein antigen
M-199: Medium-199 (Sigma)
P&S: penicillin and streptomycin
FCS: fetal calf serum
IF: immunofluorescence
PBS: phosphate-buffered saline
DMSO: dimethyl sulfoxide
α_2M: α_2-macroglobulin
SBTI: soybean trypsin inhibitor
LBTI: lima bean trypsin inhibitor
SEM: standard error of the mean
PECC: primary epidermal cell cultures
anti-AB: anti-AB blood group sera
DEAE: diethylaminoethyl ester
BAEE: benzoyl arginine ethyl ester
EDTA: ethylenediaminetetraacetic acid